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Cell Adhesion & Migration 5:4, 360-365; July/August 2011; © 2011 Landes Bioscience

Src/FAK-mediated regulation of E-cadherin as a mechanism for controlling collective cell movement

Insights from in vivo imaging

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Key words: Src, FAK, E-cadherin, EMT, fluorescent in vivo imaging

Abbreviations: ECM, extracellular matrix; EMT, epithelial to mesenchymal transition; FAK, focal adhesion kinase; AJs, adherens junctions; TGFβ, transforming growth factor beta; GFP, green fluorescent protein; FRAP, fluorescent recovery after photobleaching

Recent advances in confocal and multi-photon microscopy, together with fluorescent probe development, have enabled cancer biology studies to go beyond the culture dish and interrogate cancer-associated processes in the complex in vivo environment. Regulation of the tumor suppressor protein E-cadherin plays an important role in cancer development and progression, and may contribute to the decision between 'single cell' and 'collective invasion' in vivo. Mounting evidence from in vitro and in vivo experiments places the two non-receptor protein tyrosine kinases Src and Focal Adhesion Kinase at the heart of E-cadherin regulation and the crosstalk between integrins and cadherins. Here we discuss recent insights, attained using high-resolution fluorescent in vivo imaging, into the regulation of E-cadherin and collective invasion. We focus on the regulatory crosstalk between the Src/FAK signaling axis and E-cadherin in vivo.

Introduction

The ability of cells to invade and metastasize is linked to de-regulation of interactions with adjacent cells and surrounding extracellular matrix (ECM). The de-stabilization of cell-cell contacts and concomitant 'enrichment' of integrin-mediated cell-matrix adhesions is likely a key step in the development of cancer, and is linked to epithelial plasticity and the Epithelial to Mesenchymal Transition (EMT). The adhesion switch is often accompanied by an increase in the migratory potential of tumor cells, and may play a key role in driving tumor cell invasion.[1] However, EMT is not necessarily a pre-requisite for tumor cell invasion. Normal cells can move in a collective manner that is dependent on the maintenance of cell-cell adhesion,[2] as occurs in the well characterized phenomenon of collective cell movement in morphogenesis.[3] Understanding how de-regulation of cell-cell contact occurs, and the mechanisms that regulate collective cell movement during tumor development are important current topics in cancer research, and may lead to new anti-invasive strategies. Recent technological advances in the application of high-resolution fluorescent microscopy in vivo has enabled researchers to probe these processes in greater detail, thereby providing insights into the pathways that regulate E-cadherin, cell-cell adhesion and collective cell movement in pre-clinical cancer models. Here we review how confocal and multi-photon microscopy have added to the study of E-cadherin and collective cell invasion in vivo, including studies on regulation involving Src/Focal Adhesion Kinase (FAK) signaling.

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Submitted: 05/18/11; Accepted: 07/11/11
DOI: 10.4161/cam.5.4.17290

E-Cadherin and Cancer

E-cadherin is the prototypical member of the type-1 classical cadherins and a key component of cell-cell adhesions known as adherens junctions (AJs). This single-pass transmembrane glycoprotein facilitates cell-cell adhesion via a homophilic calcium-dependent interaction with opposing molecules on neighboring cells[4] and is commonly de-regulated in many cancers.[5] E-cadherin expression or cell surface localization is often lost in advanced tumors[5] and this loss is linked, at least in some cases, to metastasis and tumor recurrence.[6] Loss of E-cadherin in human tumors is often the result of promoter methylation or upregulation of the transcriptional repressors Snail, Slug, SIP1 or Zeb1, which target the E-cadherin promoter.[7] However, de-regulation of E-cadherin surface localization has also been linked to the activity of non-receptor tyrosine kinases that have oncogenic potential, including Src and FAK,[8,9] or the action of transforming growth factor beta (TGFβ),[10] and hepatocyte growth factor (HGF).[11]

Src/FAK Regulation of E-Cadherin

Src is the prototypical member of the Src family of non-receptor tyrosine kinases and complexes with FAK at sites of cell-matrix adhesion often termed focal adhesions or focal contacts. Elevated expression of these kinases has been reported in many tumor types, and is frequently linked to poor clinical outcome.[12-20] Several lines of evidence now support the idea of crosstalk between components of integrin adhesions and AJs, and suggest that Src/FAK signaling is at the heart of this crosstalk. For example, Src induced de-regulation of E-cadherin in colon cancer cells is dependent on integrin signaling and FAK phosphorylation.[9] In support of this, treatment of hepatocytes with the Src inhibitor PP2, or expression of the endogenous interfering version of FAK, namely FAK-related non-kinase (FRNK), is reported to inhibit TGFβ-induced de-localization of E-cadherin.[10] Taken together, these data suggest that status of the Src/FAK signaling axis controls epithelial morphology, including TGFβ-induced de-regulation of E-cadherin. Recently, we have shown that siRNA mediated depletion of FAK or β1-integrin, and inhibition of Src family kinase activity using the small molecule inhibitor *dasatinib*, causes inhibition of E-cadherin endocytosis, phenocopying effects of the dynamin inhibitor *dynasore*.[21] Furthermore, inhibition of the Src/FAK pathway using *dasatinib* and *PF-562,271*, or downregulation of FAK expression using siRNA, strengthens E-cadherin-mediated cell-cell adhesions, while overexpression of a Src Y527F mutant with constitutive kinase activity results in weakening of cell-cell adhesion strength. Using the human A431 squamous carcinoma cell line, which invades in a collective manner that is dependent on AJs integrity,[22] Src/FAK-mediated effects on E-cadherin and cell-cell adhesion strength were observed to correlate with altered E-cadherin dynamics at sites of cell-cell adhesion and inhibition of collective tumor cell invasion in vitro.[21] These data provide strong evidence that Src/FAK signaling regulates E-cadherin membrane localization and function, such that increased pathway activity de-regulates E-cadherin and drives EMT, while reduced pathway activity maintains E-cadherin membrane localization and epithelial morphology.

What is not clear is the role of Src/FAK signaling in the formation of new cell-cell adhesions. Overexpression of the gain-of-function Src Y527F mutant inhibits new junction formation in a manner that depends on FAK phosphorylation by Src.[9] This stabilizes the mesenchymal-like phenotype resulting from SrcY527F expression in colon carcinoma cells. However, in NBT-II rat bladder carcinoma cells, expression of a kinase defective FAK mutant inhibits the formation of new E-cadherin-mediated cell-cell adhesions, while overexpression of FAK itself has no effect on junction assembly.[23] These somewhat paradoxical results leave an unclear picture as to the role of Src/FAK signaling in the formation of new cell-cell adhesions, suggesting that further work is required in order to fully understand the complexity, and possibly context dependence, of AJ assembly.

In vitro studies addressing the role of Src/FAK signaling in the regulation of E-cadherin, cell-cell adhesion and collective cell migration have provided insights into the crosstalk between key components of integrin-based cell-matrix adhesions and AJs, although mechanisms are not yet fully understood. The culture dish represents a very simplistic system for studying biology and there is a drive to move toward more physiological environments. The field of high-resolution fluorescent intra-vital microscopy affords us this opportunity and has enabled researchers to study complex biological problems in a setting that is more physiologically relevant.

High Resolution Cancer Imaging in Mice

Recent advances in confocal and multi-photon microscopy, together with the development of fluorescent probes, have led to new cancer biology studies in vivo. These techniques show great promise in furthering our understanding of tumor/host interactions in the in vivo environment, and the mode of action of novel therapeutic agents. For example, using multi-photon microscopy the Condeelis lab have identified an intimate relationship between tumor cells and infiltrating macrophages, providing support for a role for macrophages in aiding tumor cell invasion and entry into blood vessels.[24] Fluorescent labeling of tumor cells with Green Fluorescent Protein (GFP), and its variants, has enabled visualization of single tumor cells either via exteriorization of the primary tumor (subcutaneous, orthotopic or genetically engineered tumor models) or through the establishment of tumors under 'optical windows.' These windows act to replace the mouse skin, which represents an impenetrable barrier that does not permit confocal and multi-photon microscopy due to contrast-robbing autofluorescence and excessive light scattering. A glass coverslip or 'optical window' provides a suitable transparent surface through which to image the tumor sample. Recently, different optical window types have been described, including the dorsal skinfold window (**Fig. 1A**),[21,25,26] the mammary window (**Fig. 1B**),[27] and the cranial window (similar design to mammary window).[26]

Aside from optical performance, these windows provide a number of other benefits. First, using anesthetics such as isofluorane, repeated imaging can be performed on a routine basis, thus facilitating the observation of tumor-associated processes over periods of days or weeks. Second, no surgery is required at the time of imaging, thereby minimising the risk of an artifactual inflammatory response that may affect tumor behavior. Third, mechanical clamping of the window to the microscope stage greatly reduces sample movement, thereby facilitating the application of advanced imaging techniques that include Fluorescence Recovery After Photobleaching (FRAP) and photoactivation to enable molecular dynamic studies deep in tumor tissue. However, optical windows also have their limitations. These include cell/tissue adhesion to the glass coverslip (although this can be overcome by careful replacement of the coverslip) and the time-consuming surgical installation of the windows, which takes longer than surgical exteriorisation of tumors for imaging. Both approaches have been used for imaging cell behavior and the surrounding microenvironment in tumor-bearing mice, providing new insights into, among other things, the regulation of E-cadherin dynamics, cell-cell adhesion and collective cell migration in vivo.

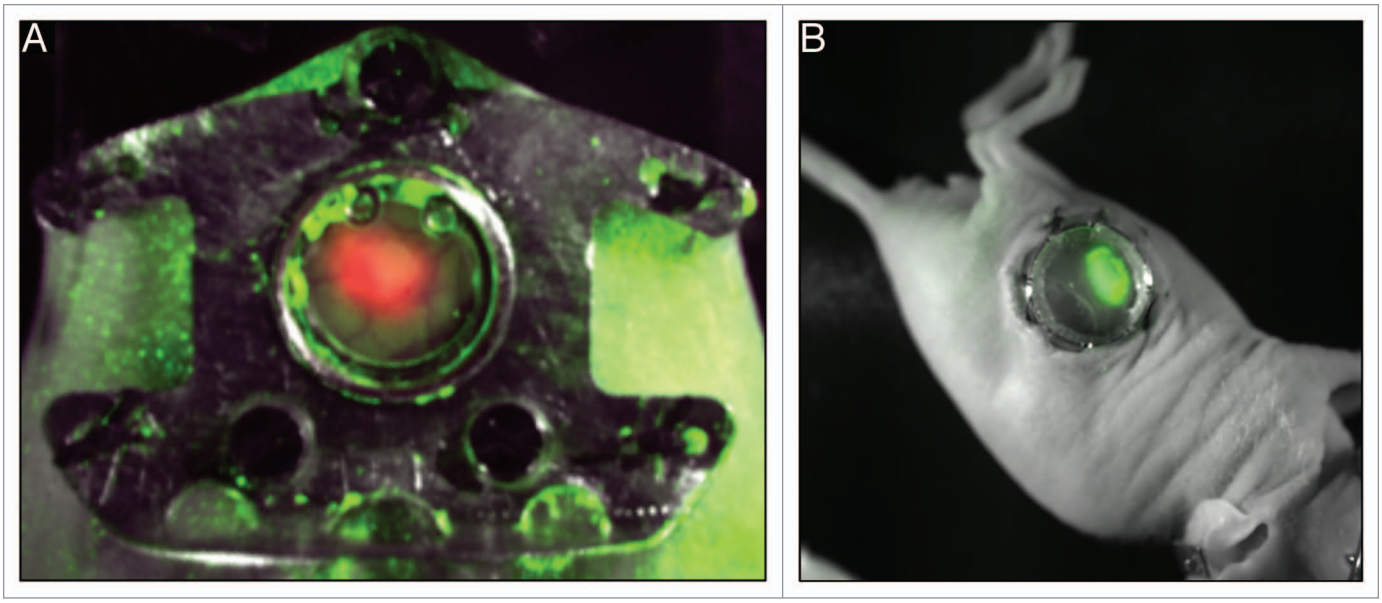

Figure 1. Images of optical window chambers. (A) tagRFP-expressing tumor under dorsal skinfold window (red), with GFP autofluorescence image (green) and brightfield overlay (gray). Cropped image acquired using 0.14x objective lens on Olympus OV110 microscope. (B) MDA-MB-231 GFP expressing tumor under mammary window (green) with brightfield overlay (gray) acquired using 0.14x objective lens on Olympus OV110 microscope.

Imaging Collective Tumor Cell Movement

The ability of tumor cells to migrate as strands or sheets in a collective manner has been reported following implantation of human tumor samples into 3D collagen lattices.[28] These experiments identify multi-cellular clusters of various sizes, which actively migrate from tissue into the surrounding collagen, suggesting that tumor cells can migrate while maintaining cell-to-cell contact. Indeed, the maintenance of functional cell-cell adhesions, and therefore physical contact with neighboring cells, is a hallmark of so called collective invasion.[29] The first demonstration of tumor cell collective invasion in vivo was recently reported by the Friedl lab.[25] These authors combined high-resolution multi-photon imaging with dorsal skin-fold windows, and identified both individually migrating cells, and two morphologically distinct patterns of collective invasion following injection of HT-1080 fibrosarcoma cells deep into the mouse dermis.[25] The authors also reported a role for surrounding tissue architecture as a determinant of the choice between singe cell invasive migration and collective invasion. Real-time imaging of MTLn3E breast tumors in vivo by the Sahai lab, has also shown the presence of both collective and singly migrating cells. Moreover, use of fluorescent reporters of TGFβ activity, such as GFP-Smad2 and $CAGA_{12}$-CFP, has demonstrated that the decision to move as a single cell or in a collective manner can be driven by localized TGFβ activation, resulting in a transient and reversible EMT.[30] Together, these studies indicate the presence of multiple factors within the tumor microenvironment that influence the mode of tumor cell invasion in vivo. Furthermore, the presence of both single and collectively invading cells within individual tumors, and the possibility of plasticity i.e., inter-conversion between these, suggests that de-regulation of E-cadherin and cell-cell adhesions may be a key determinant of invasion mode.

Further insights into the mechanisms regulating E-cadherin and cell-cell adhesion integrity in vivo comes from our recent work addressing the role of Src/FAK signaling in the regulation of E-cadherin, cell-cell adhesion and collective cell migration. We established the existence of extensive regulatory cross-talk between components of integrin-based cell-matrix adhesions and AJs in the in vivo environment, particularly E-cadherin membrane dynamics, cell-cell adhesion integrity and collective cell migration within primary tumors. We discuss this work below, introducing some principles for imaging of E-cadherin dynamics and cell tracking in an in vivo tumor context.

We first performed high-resolution confocal microscopy, focusing on the comparison of E-cadherin membrane dynamics between in vitro confluent cell monolayers and subcutaneous epithelial tumors established following injection of human A431 epidermoid squamous carcinoma cells that had been engineered ex vivo to express GFP-tagged E-cadherin. Using FRAP, we identified a molecular dynamic signature in vitro based on recovery half-time and immobile fraction measurements that correlates with the migratory capacity of cells. The rate of fluorescent recovery provides a measure of how quickly mobile molecules move into the bleached region and specifies a parameter known as the recovery half-time. This is determined by the way in which molecules move within the cell, i.e., whether they undergo simple diffusion, vesicle trafficking, or binding interactions with other molecules in the local environment.[31] Studies using GFP-E-cadherin or a photoactivatable form of GFP-E-cadherin, together with inhibitors of endocytosis, i.e., *dynasore*, suggest that half-time measurements are likely a readout of E-cadherin endocytosis, lateral movement within the plasma membrane or a combination of both.[32,33] However, the relative contribution of each is somewhat context-dependent. The cellular environment

also imposes further restrictions on mobility, such that some molecules are detained and therefore do not vacate physical space required for new unbleached molecules to occupy. Such a situation gives rise to an incomplete recovery when compared with the initial fluorescent signal and when measured is termed the immobile fraction.[31] We concluded that non-motile cells exhibit a faster recovery half-time and a higher immobile fraction. Comparison of E-cadherin dynamics at sites of cell-cell adhesion between cell cultures in vitro and tumors in vivo identified significant differences between these environments, both in terms of basal dynamics and response to therapeutic treatment with the Src kinase inhibitor *dasatinib*. Treatment of tumor-bearing mice with *dasatinib* alters E-cadherin dynamics in a manner indicative of reduced migratory capacity,[34] although quantitative measurement of the true migratory behavior of these cells was not determined. Surgical exposure of the tumor as a skin-flap in these experiments restricted the quantification of migratory behavior, and also provided challenges in terms of minimising focal drift of the sample in the x, y and z axis required for techniques such as FRAP and photoactivation.

Switching of our experimental approach from skin-flaps to dorsal skin-fold windows greatly reduced sample movement during image acquisition, at the same time allowing us to use recoverable anesthesia to perform long-term tracking of tumor cell behavior over multiple imaging sessions.[21] Similar approaches have been described elsewhere in references 25–27. When coupled with photoswitchable fluorescent proteins, this provides a means of tracking sub-populations of tumor cells over a period of 24–48 h.[21,27] Quantitative measurement of A431 collective cell migration in vivo using this approach revealed that inhibition of either FAK or Src kinase activity (using the kinase inhibitors *PF-562,271* and *dasatinib* respectively), results in inhibition of cell migration within the primary tumor (**Fig. 2A**),[21] thereby targeting a process that is likely to be required for the intra-vasation and dissemination of tumor cells from the primary tumor. Moreover, identification of multi-cellular strands some distance from the original activation point suggested the presence of collectively migrating cells within these tumors (**Fig. 2B**), although this cannot be definitively proved using an assay based on the acquisition of still images at any given time. While individual labeled cells are also visible, we cannot tell whether these were singly migrating cells or cells migrating in a collective manner with cells that had not been switched from green to red. This assay format does not permit the accurate quantification of singly and collectively migrating cells, but it does allow quantification and visualization of what appears to be collective migration. The photoswitching of Dendra2-labeled tumor cells through mammary windows has also been used for measurement of tumor cell entry into blood vessels, with the sub-population of cells being selected for their proximity to a blood vessel.[27] This approach established that Src/FAK signaling is required for the collective migration of tumor cells in vivo, and also provided pre-clinical evidence that *dasatinib* and *PF-562,271* display anti-invasive effects (**Fig. 2A**).[21]

To investigate the mechanism by which Src/FAK signaling was regulating collective cell migration in vivo, we used FRAP in GFP-tagged E-cadherin-expressing tumor cells grown under optical windows. Treatment of tumors with *PF-562,271* resulted in a shorter recovery half-time and no change in immobile fraction, mimicking the effects of *PF-562,271* treatment in cells in culture. Further investigation in vitro revealed that these dynamic changes in E-cadherin mobility at sites of cell-cell adhesion correlate with inhibition of E-cadherin endocytosis, inhibition of collective invasion into a 3D collagen matrix and strengthening of cell-cell adhesion. In addition, these studies confirm that GFP-E-cadherin recovery half-time measurements are at least in part a measure of the rate of E-cadherin endocytosis.[21] These imaging studies provide new insights into the existence of regulatory cross-talk between components of integrin-based cell-matrix adhesions and AJs in vivo, while further identifying potential mechanisms underlying the potential anti-invasive effects of small molecule tyrosine kinase inhibitors targeting Src/FAK signaling in epithelial tumors. However, these studies do not rule out the possibility that inhibition of Src/FAK signaling may have a general effect on cell motility, independent of E-cadherin and AJs status, that may contribute to the potential anti-invasive effects of Src/FAK inhibition in epithelial tissue.

Conclusions and Future Perspectives

To date very few studies have investigated the role of Src/FAK signaling in the cancer-associated regulation of E-cadherin and cell-cell adhesion by imaging in the in vivo setting. In vitro cell culture can at best only partially re-capitulate the in vivo environment; new techniques allow this to be overcome with the use of high-resolution imaging in vivo. Indeed, in vivo confocal and multi-photon microscopy has highlighted the heterogeneity of tumor cell invasion modes, even within individual tumors,[25,30] and indicated that localized signaling gradients in primary tumors can induce transient and reversible EMT.[30] Such insights could not have been gained from purely in vitro studies. Evidence emerging from our own in vivo studies investigating the role of Src/FAK signaling in E-cadherin and cell-cell adhesion regulation implies that inhibition of this signaling pathway strengthens cell-cell adhesion in vivo,[21] promoting a more epithelial-like morphology and suppressing epithelial tumor cell migration. As expected, the biology in vivo is complex and we hypothesize that inhibition of Src/FAK signaling may counteract the pro-EMT signals within the tumor microenvironment, such as those induced by TGFβ, therefore reducing the movement within and away from the tumor. Studies addressing the mechanisms that underpin tumor cell responses to small molecule inhibitors in vivo are therefore important in testing putative anti-cancer therapies. High-resolution in vivo imaging in the pre-clinical setting will aid understanding of tumor/host interplay and predicting responses to therapeutic intervention. New quantitative probes for cancer biology imaging in vivo, particularly if these can be applied to more relevant cancer models (genetically engineered), will facilitate further progress.

Acknowledgments

The authors would like to acknowledge funding from Cancer Research UK, program grant C157/A9148.

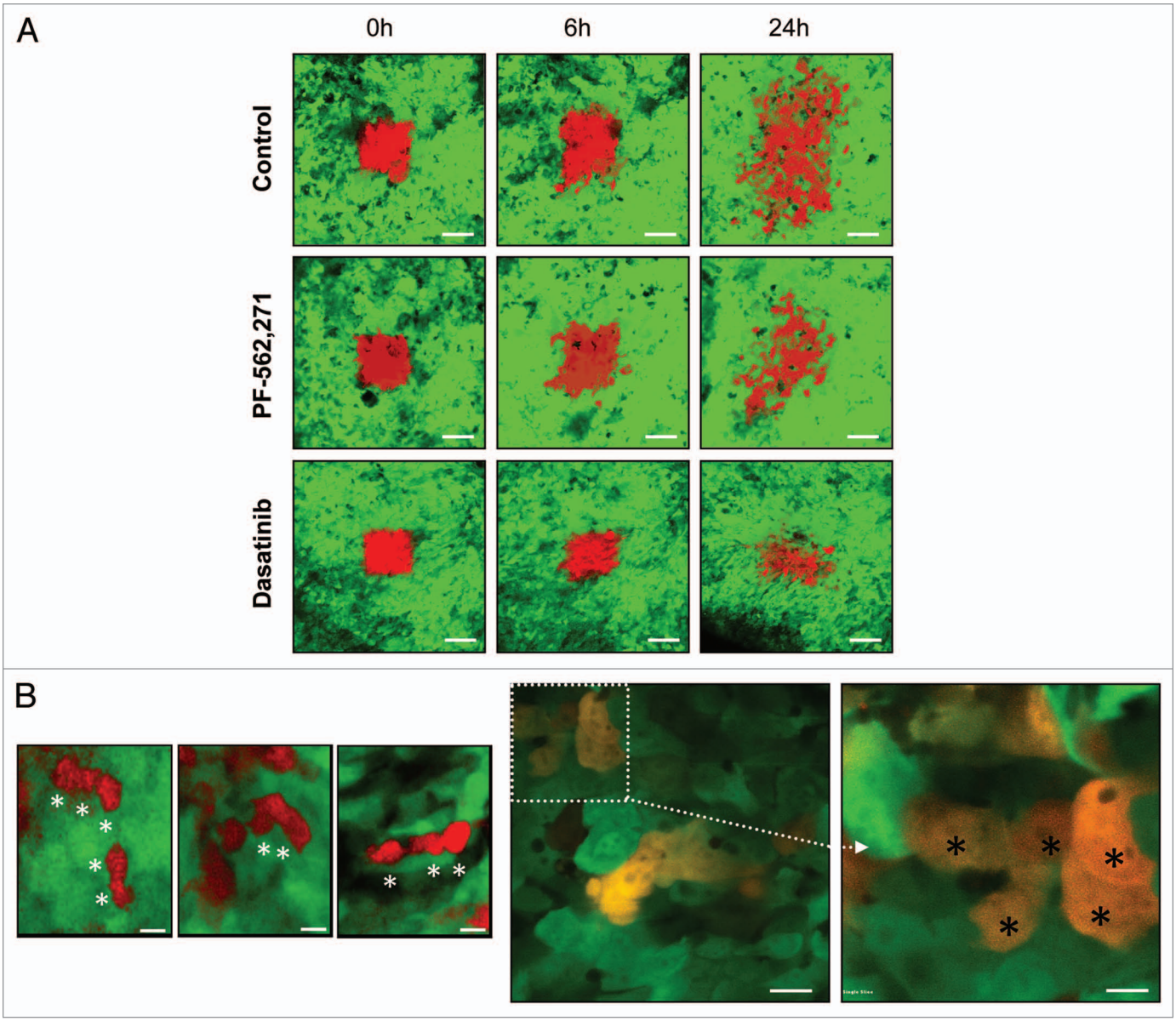

Figure 2. Visualizing collective cell migration in living animals. (A) Confocal images showing A431 Dendra2 cells in tumors of untreated mice or mice treated with PF-562,271 or dasatinib, at different time points after photoswitching (red). Scale bars, 100 μm. (B) Zoomed images from a control tumor (left) and higher magnification images (center and right) showing collective cell movement. *individual cells within a group; scale bars, 20 μm (left and center images) and 10 μm (right image). All images presented in this figure have been previously published in reference 21.

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